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(57) Abstract

The present invention provides methods of using a composition comprising a hydroxybenzoate metabolite, a hydroxybenzoate analogue, or a mixture thereof in the prophylactic and therapeutic treatment of a biological material, including, but not limited to, a cell, a tissue, an organ and an organism, for, among others, oxidative stress and chemotherapeutic drug toxicity, cardiotoxicity and extravasation tissue injury.

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HYDROXYBENZOATE METABOLITES AND HYDROXYBENZOATE ANALOGUES AS PROTECTORS AGAINST OXIDATIVE STRESS AND CHEMOTHERAPEUTIC DRUG CYTOTOXICITY, CARDIOTOXICITY AND EXTRAVASATION TISSUE INJURY

FIELD OF THE INVENTION

The present invention relates to methods of using a composition comprising a hydroxybenzoate metabolite, a hydroxybenzoate analogue, or a mixture thereof in the prophylactic and therapeutic treatment of a biological material, including, but not limited to, a cell, a tissue, an organ and an organism, for, among others, oxidative stress and chemotherapeutic drug cytotoxicity, cardiotoxicity and extravasation tissue injury.

BACKGROUND OF THE INVENTION

Although oxygen is required to sustain most of life on Earth, during the metabolism of oxygen, a variety of toxic oxygen-related species, such as hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2) and superoxide (O_2^-), are produced, which, if left unchecked, can undoubtedly damage cells. Cells, however, have evolved elaborate detoxification and repair systems to rid themselves of these potentially toxic and undesirable metabolic by-products. For example, superoxide dismutase (SOD) can convert superoxide to H_2O_2, and catalase (CAT) can convert H_2O_2 to H_2O.

Hydrogen peroxide (and organoperoxides) also can be detoxified by glutathione peroxidase (GPX), which, with glutathione (GSH), converts H_2O_2 to H_2O. Glutathione transferase (GST), in addition to its ability to conjugate and inactivate drugs and xenobiotics, possesses peroxidase activity and, thus, can also detoxify H_2O_2. These systems represent the major detoxification pathways for oxygen-derived free radical species. There are undoubtedly other systems that may provide protection, such as protein sulfhydryls and thiol-related enzymes, which could be involved in repair mechanisms.

Despite the efficiency of these enzymatic systems, there is a small "leakage" of toxic species beyond the biochemical defense network. Of particular importance is the ultimate fate of H_2O_2 should it escape detoxification. For example, H_2O_2 can undergo reduction via ferrous complexes to produce the highly reactive •OH, which, in the order of 10^{-9} seconds, can: 1) abstract electrons from organic molecules; 2) break chemical bonds; 3) initiate lipid peroxidation; and 4) react with another •OH to produce H_2O_2.

It is not known whether chronic exposure to low levels of oxygen-derived free radical species is deleterious. It is postulated, however, that the process of aging may be a manifestation of the organism's inability to cope with sustained oxidative stress.

In addition, many cancer treatment modalities, including x-rays, other forms of radiation therapy, and some chemotherapeutic drugs, exert their cytotoxicity via production of oxygen-related free radicals, thereby imposing an added burden to normal detoxification systems. Furthermore, free radicals and toxic oxygen-related species have been implicated in ischemia reperfusion injury, stroke, allergies, amyotrophic lateral sclerosis (ALS), and Parkinson's disease, and have long been thought to be important in neutrophil-mediated toxicity of foreign pathogens. Free radical damage also has been implicated in carcinogenesis. Accordingly, the term "oxidative stress" has emerged to encompass a broad variety of stresses, some of which have obvious implications for health care.

Although the administration of inherent intracellular detoxifying agents would appear to be useful in the protection of living cells, tissues, organs and organisms from oxidative stress, some such agents, e.g., superoxide dismutase and catalase, are impermeable. Accordingly, there has been considerable interest in devising additional approaches, apart from inherent intracellular detoxification systems, to protect cells, tissues, organs, and organisms, including animals and humans, from the toxic effects of any agent or process that imposes oxidative stress. In the past few years, experimental studies have indicated that enzymes, such as

catalase and superoxide dismutase, and agents, such as allopurinol and metal-chelating compounds, afford protection against oxidative stress. At present, none of these approaches is being applied to humans.

It is an object of the present invention to overcome the problems associated with the use of impermeable enzymatic detoxifying agents, such as superoxide dismutase and catalase, to protect biological material including a cell, a tissue, an organ and an organism, from the deleterious effects of toxic products generated during oxygen metabolism.

Another object of the present invention is to address the toxicity problems, including cytotoxicity and cardiotoxicity, associated with the use of cancer chemotherapeutic agents.

A further object of the present invention is to protect against tissue injury that results from extravasation during intravenous administration of a chemotherapeutic agent.

These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

SUMMARY OF THE INVENTION

The present invention provides methods of using a composition comprising a hydroxybenzoate metabolite, a

hydroxybenzoate analogue, or a mixture thereof, in the prophylactic and therapeutic treatment of a biological material, including, but not limited to, a cell, a tissue, an organ and an organism, for, among others, oxidative stress and chemotherapeutic drug cytotoxicity, cardiotoxicity and extravasation tissue injury.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A and 1B are bar graphs of surviving fraction of cells versus compounds, which show the survival of Chinese hamster V79 lung fibroblasts treated with 0.5 mM hypoxanthine/0.08 U/ml xanthine oxidase (HX/XO) for 45 min, in the absence or presence of one of the following: 2,3-dihydroxybenzoic acid (2,3 DHB); salicylic acid (SA); benzoic acid (BA); 2,6-dihydroxybenzoic acid (2,6 DHB); dihydroxybenzoic acid (3,4 DHB); 2,5-dihydroxybenzoic acid (2,5 DHB); 3,5-dihydroxybenzoic acid (3,5 DHB); acetyl salicylic acid (A); and 3-hydroxybenzoic acid (3 HB), and wherein, in Panel A, the concentration of compounds is 5 mM, and in Panel B, the concentration of compounds is 25 mM. Compounds were added to cells 1 hr before xanthine oxidase treatment.

FIGURE 2 is a bar graph of surviving fraction of cells versus hydroxybenzoate metabolite, which shows the survival of V79 cells treated with 1 mM hydrogen peroxide (H_2O_2) for 60 min, in the absence or presence of one of

the following: salicylic acid (SA); benzoic acid (BA); 2,3-dihydroxybenzoic acid (2,3 DHB); 2,6-dihydroxybenzoic acid (2,6 DHB); 3,4-dihydroxybenzoic acid (3,4 DHB); 2,5-dihydroxybenzoic acid (2,5 DHB); 3,5-dihydroxybenzoic acid (3,5 DHB); and acetyl salicylic acid (A). Compounds were added to cells 1 hr before xanthine oxidase treatment.

FIGURES 3A and 3B are bar graphs of surviving fraction of cells versus compounds, which show the survival of V79 cells treated with 3 mM *t*-butyl hydroperoxide (TBH) for 2 hr, in the absence or presence of one of the following: 2,3-dihydroxybenzoic acid (2,3 DHB); salicylic acid (SA); benzoic acid (BA); 2,6-dihydroxybenzoic acid (2,6 DHB); 3,4-dihydroxybenzoic acid (3,4 DHB); 2,5-dihydroxybenzoic acid (2,5 DHB); 3,5-dihydroxybenzoic acid (3,5 DHB); acetyl salicylic acid (A); and 3-hydroxybenzoic acid (3HB), and wherein the concentration of compounds in Panel A is 5 mM and the concentration of compounds in Panel B is 25 mM. Compounds were added to cells 1 hr before *t*-butyl hydroperoxide treatment.

FIGURES 4A and 4B are graphs of surviving fraction of cells versus adriamycin concentration (μg/ml)or iminodaunomycin concentration (μg/ml), respectively, which show the survival of V79 cells exposed to adriamycin or iminodaunomycin, respectively, for 1 hr in the presence or absence of 25 mM 3,4-dihydroxybenzoic

acid (DHB). DHB was added 1 hr before adriamycin or iminodaunomycin.

FIGURE 5 is a graph of % DNA remaining in well versus adriamycin concentration (μg/ml), which shows the inhibition of DNA damage as measured by pulsed-field electrophoresis of DNA from V79 cells treated with adriamycin in the presence or absence of 25 mM 3,4-dihydroxybenzoic acid (DHB).

FIGURE 6 is a graph of % DNA remaining in well versus DHB concentration (mM), which shows the dose response for protection from adriamycin-induced DNA double-strand breaks by 3,4-dihydroxybenzoic acid (DHB). Adriamycin treatment was 5 μg/ml for 1 hr.

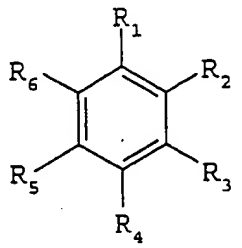
FIGURE 7 is a graph of relative surviving fraction versus etoposide (VP-16; μg/ml), which shows the survival of V79 cells treated with VP-16 in the absence or presence of 1 mM, 5 mM or 25 mM 3,4-dihydroxybenzoic acid (DHB).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides methods of using a composition comprising a hydroxybenzoate metabolite, a hydroxybenzoate analogue, or a mixture thereof, in the prophylactic and therapeutic treatment of a biological material, including, but not limited to, a cell, a tissue, an organ and an organism, for oxidative stress

and chemotherapeutic drug cytotoxicity, cardiotoxicity and extravasation tissue injury.

Hydroxybenzoate metabolites and hydroxybenzoate analogues useful in the methods of the present invention have the formula:



wherein R_1 = COO- or a precursor to COO-,

R_2 and R_3 = H, OH, or a precursor to OH, and

R_4, R_5 and R_6 = H, OH, a precursor to OH or a macromolecule.

Examples of a "precursor to COO-" include a cyano group, CH_2OH, CHO, COOR (wherein R = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule, such as a protein (in which case binding to the protein is via CH_2OH of a threonine or a serine), a lipid, a polysaccharide, a lipopolysaccharide, or a nucleic acid derivative), or an amide, such as CONR1 or CONR1R2 (wherein R1 and R2 = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule, such as a protein (in which case binding to the protein is via an ε-amino group of lysine or a direct amide cap), a lipid, a polysaccharide, a lipopolysaccharide or a nucleic acid derivative).

Examples of a "precursor to OH" include an ester and OR, wherein R is a C_{1-22} alkyl or a C_{1-22} aryl.

Examples of macromolecules for R4, R5 and R6 include a protein, a polysaccharide, a lipid, a lipopolysaccharide, and a nucleic acid derivative. R4, R5 and R6 can be part of a ring structure, such as a C_{1-22} alkyl or aromatic ring.

Examples of hydroxybenzoate metabolites include 2,3-dihydroxybenzoic acid, salicylic acid, benzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, acetyl salicylic acid, and 3-hydroxybenzoic acid.

Examples of hydroxybenzoate analogues include monohydroxybenzoates, dihydroxybenzoates and trihydroxybenzoates.

With respect to the above, it is preferred that, when R1 = C00- and R2 = OH or a precursor to -OH, wherein said precursor to OH is $CH_3C(0)0$-, at least one of R3-R6 is other than hydrogen.

The use of hydroxybenzoate metabolites and hydroxybenzoate analogues to treat oxidative stress; etc., is advantageous over aspirin for a number of reasons, including avoidance of gastric and duodenal ulcers and platelet function disorders, and the ability to realize high concentrations of specific hydroxybenzoate metabolites and/or hydroxybenzoate analogues, which can not be realized when relying on the normal metabolism of aspirin.

In addition to protecting against oxidative stress, hydroxybenzoate metabolites and hydroxybenzoate analogues are useful to protect biological material, such as a cell, a tissue, an organ and an organism, from the cytotoxic effects of cancer chemotherapeutic agents, such as anthracyclines, including, for example, adriamycin, iminodaunomycin and daunomycin, and epipodophlotoxins, including, for example, VP-16 (etoposide) and VM-26.

High doses of anthracyclines and epipodophlotoxins are given to patients for the treatment of cancer. Adriamycin is one of the most effective and widely used chemotherapeutic agents against a variety of epidermoid/sarcomatoid/adenomatoid cancers. However, the effectiveness of adriamycin is limited by adriamycin-mediated bone marrow suppression, gastrointestinal damage, and cumulative dose-dependent irreversible cardiotoxicity.

While the exact mechanism of adriamycin cytotoxicity is not known, several mechanisms have been proposed, including intercalation into DNA, stabilization of the topoisomerase II-DNA complex, and free radical-mediated toxicity caused by redox cycling of the semiquinone radical. Some data suggest adriamycin may redox-cycle in cells to produce superoxide, hydrogen peroxide, and hydroxyl radicals. In keeping with the invention, it has been found that hydroxybenzoate metabolites protect against cytotoxicity resulting from iminodaunomycin, an

anthracycline analogue of adriamycin that does not generate free radicals.

In this regard, and in accordance with the present invention, hydroxybenzoate metabolites and hydroxybenzoate analogues are also useful in the treatment of chemotherapeutic drug extravasation injury. Extravasation occurs when a hypodermic injection, such as an injection of a chemotherapeutic agent, misses the vein, thereby causing release of a high localized concentration of the chemotherapeutic agent into the surrounding tissue, which results in cellular damage and, ultimately, a disfiguring ulceration. A composition comprising a hydroxybenzoate metabolite, a hydroxybenzoate analogue, or a mixture thereof, in accordance with the present invention, can be rapidly injected at the site of the missed injection to alleviate or prevent this damage by providing a high, localized concentration of the hydroxybenzoate metabolite or analogue.

Extravasation during intravenous administration of chemotherapeutic antineoplastics has been estimated to occur between 0.1 to 6% of patients. The factors that influence the degree of tissue damage include the vesicant nature of the extravasated drug and the concentration of the extravasated agent, as well as the volume that infiltrates into the tissue. By way of illustration, several mechanisms have been proposed to

cause the severe damage by anthracycline analogues, such as adriamycin and daunomycin, as well as mitomycin, mitoxanthrone, and others. For the anthracyclines, it is known that, after extravasation, the drug is retained in the surrounding tissue for a period of months. It has been postulated that fragmented DNA-anthracycline breakdown products are released from dead cells and taken up by adjacent cells and that the products are redox active. The result is continued futile redox cycling. The histological consequence of such cycles resulting from extravasation is damage of vessels and, rarely, inflammatory cell infiltration. The gross manifestation can be a large and disfiguring ulceration in and around the site of extravasation. The consequences of the non-healing ulceration include infections, dysfunction of the affected extremity, and, sometimes, death. Oftentimes, the extravasation requires surgical debridment and skin grafting.

In accordance with a further aspect of the present invention, hydroxybenzoate metabolites and hydroxybenzoate analogues are useful to protect a cardiac cell, tissue, or organ, including a cell, tissue or organ as part of an intact living organism, from cardiotoxicity, also known as cardiac toxicity, which can result from therapeutic intervention with a number of cancer chemotherapeutic agents. Adriamycin (doxorubicin) and daunomycin, two red pigmented antibiotics

(rhodomycins) isolated from the soil bacillus Streptomyces, are most clearly associated with cardiac toxicity. Each is a polycyclic aromatic compound containing a quinone structure. The major mode of anticancer activity is thought to derive from anthracycline inhibition of topoisomerase II activity. Other mechanisms of activity include DNA intercalation and redox cycling. The underlying mechanism of cardiac toxicity is not well-established. Generally, anthracyclines exhibit three forms of cardiac toxicity.

In the first form of cardiac toxicity, acute toxicity occurs within hours and manifests as supraventricular tachyarrhythmias, which are readily demonstrable by electrocardiography. Rarely, a more lethal ventricular dysrhythmia may occur. Although the incidence of ectopy may be higher than 90%, the arrhythmias are transient for both drugs and do not seem to be dose- or schedule-dependent, and, more importantly, do not seem to be associated with the subsequent development of cardiomyopathy.

The second form of cardiac toxicity, subacute toxicity, is mostly seen with daunomycin and occurs up to weeks after administration of the drug, manifesting as either myocarditis or pericarditis. Once again, the subacute toxicity appears to be idiopathic and is not associated with dose or schedule of delivery.

In the third form of cardiac toxicity, chronic effects occur weeks to months and, in some cases, years following administration of the drug, are closely correlated with cumulative dose, and may lead to congestive heart failure and death. Chronic toxicity is a major toxicity and is overwhelmingly the most important cardiac toxicity associated with clinical anthracycline usage. The risk associated with cumulative dose, although not completely absent, remains exceedingly low until doses of greater than 500 mg/M^2, whereupon the risk of developing cardiomyopathy rises linearly as a function of continued treatment. The exact cause of the cardiotoxicity is unknown, although redox cycling-induced cellular dysfunction, calcium channel alteration within the sarcoplasmic reticulum, mitochondrial cardiolipin association and electron cascade disruption, and intercalation all have been claimed. Whatever the molecular mechanism of cardiac toxicity, the histopathologic picture is the same in numerous animal models, including man. There are acute changes, which include hypereosinophilia of myocytes, accumulation of hyaline material, contraction bands, and cytoplasmic granulation, and there may be infiltration around the myocyte with inflammatory neutrophils. The chronic changes occur in a systematic and predictable fashion characterized as myofibrillar dropout, i.e., the loss of the contractive elements of the myocytes.

In addition to doxorubicin and daunomycin, which have traditionally been most extensively used in clinical medicine, other anthracyclines include epirubicin, idarubicin, esorubicin, aclarubicin, and menogaril. Furthermore, other redox-cycling drugs include bleomycin, mitoxanthrone (novatrone), losoxanthrone, mitomycin C, and actinomycin. Additionally, a number of alkylating agents have caused cardiotoxicity, when given after anthracyclines or radiation, and they include ifosfamide, carmustine (BCNU), cyclophosphamide, busulfan (endocardial fibrosis), cisplatin and carboplatin, as well as nitrogen mustard. Amsacrine (m-AMSA), which ostensibly functions as an antineoplastic agent by intercalation, has similar cardiac toxicity to that of anthracycline antibiotic antineoplastics.

Accordingly, the present invention provides a method for therapeutically or prophylactically treating the effects of oxidative stress due to the production of harmful free radical species, cytotoxicity resulting from therapeutic intervention with a cancer chemotherapeutic agent, cardiotoxicity resulting from therapeutic intervention with a number of cancer chemotherapeutic agents, or chemotherapeutic drug extravasation injury. This method comprises administering a composition comprising an anti-oxidative stress, anti-cytotoxicity, anti-cardiotoxicity or anti-chemotherapeutic drug extravasation injury effective amount, respectively,

e.g., a prophylactically or therapeutically effective amount, of an aforesaid hydroxybenzoate metabolite, hydroxybenzoate analogue, or a mixture thereof, to a biological material, such as a living cell, tissue, organ or organism susceptible to oxidative stress, cytotoxicity, cardiotoxicity, or chemotherapeutic drug extravasation injury, respectively.

Preferred hydroxybenzoate metabolites for use in the present inventive methods are 3,4-dihydroxybenzoic acid and 2,3 dihydroxybenzoic acid. Preferably, the hydroxybenzoate metabolite, hydroxybenzoate analogue or mixture thereof is administered to the biological material, such as a cell, tissue, organ or organism, as part of a composition. Suitable compositions are known to those of skill in the art. More preferably, the composition comprises a biologically acceptable carrier as is known to those of skill in the art.

The composition can be administered by any one of a number of routes, such as orally, through pills or elixirs, for example, or topically, through an ointment, lotion or cream, for example, for treatment of oxidative stress; or orally, intravenously or intra-arterially, for example, for treatment of cytotoxicity or cardiotoxicity; or topically, through application as an ointment, lotion or cream, for example, orally, intravenously, systemically, or locally, by direct injection to or around the injured site, for example, for

chemotherapeutic drug extravasation injury. Liposomal formulations can be useful, in particular with respect to chemotherapeutic drug extravasation injury.

The compositions also can be administered to treat prophylactically or therapeutically reperfusion injury due to myocardial infarction or stroke, for example, pancreatitis, intestinal ulceration, and organ transplants (including use in organ preservation solutions), as well as oxygen-induced lenticular degeneration and hyaline membrane disease in infants and oxidative stress associated with oxygen therapy or hyperbaric oxygen treatment.

The dosage of a hydroxybenzoate metabolite, hydroxybenzoate analogue or mixture thereof to be administered to a cell, tissue, organ or organism should be sufficient to achieve the desired effect, i.e., an "effective amount." The dose administered will vary with the composition, route of administration, condition being treated, and the biological material/site being treated.

EXAMPLES

The following examples further illustrate the present invention and, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

Protection of Cells Against Oxidative Damage

The aspirin metabolites used in Example I were obtained from commercial sources, such as Sigma Chemical Co., St. Louis, MO.

Cell lines were maintained in a 5% CO_2/95% air humidified atmosphere at 37°C. Chinese hamster V79 lung fibroblasts (ATCC, Rockville, MD) were grown in Ham's F12 medium with 10% heat-inactivated fetal bovine serum and antibiotics. One day before each experiment, 5 X 10^5 cells were plated into 100 mm Petri dishes and incubated overnight. Various aspirin metabolites and analogues was dissolved in Ham's F12 medium at a final concentration of 5 or 25 mM, and added to the cells 1 hr prior to a 1 hr-treatment with 1 mM hydrogen peroxide, a 2 hr-treatment with 3 mM tert-butyl hydroperoxide, or a 45 min exposure to hypoxanthine/xanthine oxidase (0.5 mM/0.08 U/ml). After drug treatment, the cells were rinsed, trypsinized, and plated for clonogenic survial. Colonies were fixed and stained with crystal violet after 6-7 days incubation.

As shown in Figures 1-3, selected benzoates provided significant protection against the cytotoxicity mediated by hydrogen peroxide, superoxide generated from hypoxanthine/xanthine oxidase, or tert-butyl

hydroperoxide. The best protectors were 3,4 DHB and 2,3 DHB.

EXAMPLE 2

Protection of Cells Against Chemotherapeutic Drug Toxicity

3,4-dihydroxybenzoic acid (3,4 DHB), commonly known as protocatechuic acid, was obtained from Sigma Chemical Co., St. Louis, MO, and Adriamycin® was obtained from Pharmacia, Columbus, OH. VP-16 (etoposide) and iminodaunomycin were obtained from the National Cancer Institute Drug Synthesis and Chemistry Branch.

Cell lines were maintained in a 5% CO_2/95% air humidified atmosphere at 37°C. Chinese hamster V79 lung fibroblasts were grown in Ham's F12 medium with 10% heat-inactivated fetal bovine serum and antibiotics. One day before each experiment, 5 X 10^5 cells were plated into 100 mm Petri dishes and incubated overnight. 3,4 DHB was dissolved in F12 medium and added to the cells 1 hr prior to a 1 hr-treatment with adriamycin, iminodaunomycin or VP-16. After drug treatment, the cells were rinsed, trypsinized, and plated for clonogenic survival. Colonies were fixed and stained with crystal violet after 6-7 days incubation.

Cells for electrophoresis were plated as described above, and the DNA was labeled by incubating the cells with 0.02 μCi/ml ^{14}C-thymidine for 24 hrs prior to

adriamycin treatment. DNA was prepared for electrophoresis and field inversion electrophoresis was performed. The data are expressed as "% DNA remaining in the well" and calculated as follows:

$$\% \text{ DNA remaining} = \frac{\text{CPM in the well}}{\text{CPM in the lane + CPM in the well}} \times 100$$

The catechol derivative 3,4-dihydroxybenzoic acid (3,4 DHB) is known to scavenge hydroxyl radicals. The ability of 3,4 DHB to protect against adriamycin cytotoxicity and DNA double-strand break induction in the V79 cells was investigated. Because of the possibility of topoisomerase II involvement in adriamycin toxicity, the effect of DHB on topoisomerase I and II activity *in vitro* was also investigated.

Topoisomerase assays were done using TopoGEN, Inc., Topoisomerase I and II assay kits, according to protocols provided by TopoGEN (Columbus, OH). Substrates for the two topoisomerases were supercoiled plasmid DNA (scDNA) and trypanosome catenated kinetoplast DNA (KDNA), respectively. Reaction products were electrophoresed in 1% agarose gels for 30 min in 0.5X TAE at 100 V. Gels were stained with 0.5 μg/ml ethidium bromide for 30 min and destained for 30 min in distilled water.

Figure 4 shows clonogenic survival for V79 cells treated with adriamycin or iminodaunomycin in the presence or absence of 25 mM 3,4 DHB. 3,4 DHB provides

considerable protection against adriamycin or iminodaunomycin cytotoxicity, with dose modifying factors of 3.5 and 2, respectively, at 1% survival. Figure 5 shows the results of pulsed field gel electrophoresis experiments, which detect DNA double-strand breaks. 25 mM 3,4 DHB reduced the amount of DNA migrating out of the wells, indicating that it protected against induction of double-strand DNA breaks by adriamycin. Figure 6 shows a dose response curve for 3,4 DHB in cells treated with 5 μg/ml adriamycin. Increasing doses of 3,4 DHB provided increasing protection against adriamycin-induced DNA damage.

3,4 DHB inhibits topoisomerase II nearly completely at 25 mM and, to a lesser extent, at lower doses, while it does not inhibit topoisomerase I.

Figure 7 shows that 3,4 DHB also protects against the cytotoxicity of a different chemotherapeutic agent, namely VP-16 (etoposide), which is thought to also exert cytotoxicity by inhibition of topoisomerase II.

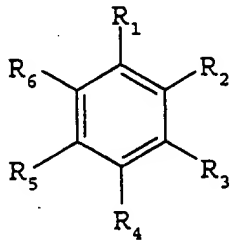
All publications cited herein are hereby incorporated by reference to the same extent as if each publication were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

While this invention has been described with emphasis upon preferred embodiments, it will be obvious

to those of ordinary skill in the art that the preferred embodiments may be varied. It is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of prophylactically or therapeutically treating a cell, a tissue, an organ or an organism for oxidative stress, which method comprises administering to said cell, tissue, organ or organism a composition comprising an anti-oxidative stress effective amount of a compound of formula:



wherein R_1 = COO- or a precursor to COO-,

R_2 and R_3 = H, OH, or a precursor to OH, and

R_4, R_5 and R_6 = H, OH, a precursor to OH or a macromolecule or, together, R_4,R_5 and R_6 are part of a ring structure, with the proviso that, when R_1 = COO- and R_2 = OH or a precursor to OH, wherein said precursor to OH is $CH_3C(O)O$-, at least one of R_3-R_6 is other than H.

2. The method of claim 1, wherein said precursor to COO- is selected from the group consisting of (i) a cyano group, (ii) CH_2OH, (iii) CHO, (iv) COOR, wherein R = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via a threonine or a serine), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid

derivative, and (v) an amide, wherein said amide is selected from the group consisting of CONR1 or CONR1R2, wherein R1 and R2 are selected from the group consisting of a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via an ε-amino group of lysine or a direct amide cap), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative.

3. The method of claim 1, wherein said precursor to OH is selected from the group consisting of an ester and OR, wherein R is a C_{1-22} alkyl or a C_{1-22} aryl.

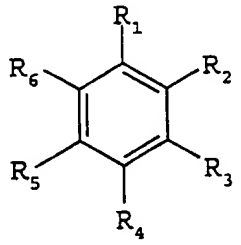
4. The method of claim 1, wherein, when R4, R5 or R6 is a macromolecule, the macromolecule is selected from the group consisting of a protein, a polysaccharide, a lipid, a lipopolysaccharide, and a nucleic acid derivative.

5. The method of claim 1, wherein, when R4, R5 and R6 are part of a ring structure, the ring structure is a C_{1-22} alkyl ring or a C_{1-22} aromatic ring.

6. The method of claim 1, wherein said compound is selected from the group consisting of a monohydroxybenzoate, a dihydroxybenzoate and a trihydroxybenzoate.

7. The method of claim 1, wherein said compound is selected from the group consisting of 2,3-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid.

8. A method of prophylactically or therapeutically treating a cell, a tissue, an organ or an organism for cytotoxicity, which method comprises administering to said cell, tissue, organ or organism a composition comprising an anti-cytotoxicity effective amount of a compound of formula:



wherein R_1 = COO- or a precursor to COO-,

R_2 and R_3 = H, OH, or a precursor to OH, and

R_4, R_5 and R_6 = H, OH, a precursor to OH or a macromolecule or, together, R_4, R_5 and R_6 are part of a ring structure, with the proviso that, when R_1 = COO- and R_2 = OH or a precursor to OH, wherein said precursor to OH is $CH_3C(O)O$-, at least one of R_3-R_6 is other than H.

9. The method of claim 8, wherein said precursor to COO- is selected from the group consisting of a (i) cyano group, (ii) CH_2OH, (iii) CHO, (iv) COOR, wherein R = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via a threonine or a serine), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative, and (v) an amide, wherein said amide is selected from the group consisting of CONR1 or CONR1R2, wherein R1 and R2 are selected from the group consisting of a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via an ε-amino group of lysine or a direct amide cap), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative.

10. The method of claim 8, wherein said precursor to OH is selected from the group consisting of an ester and OR, wherein R is a C_{1-22} alkyl or a C_{1-22} aryl.

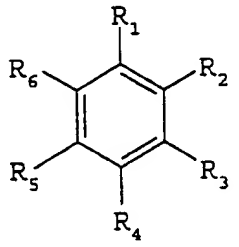
11. The method of claim 8, wherein, when R4, R5 or R6 is a macromolecule, the macromolecule is selected from the group consisting of a protein, a polysaccharide, a lipid, a lipopolysaccharide, and a nucleic acid derivative.

12. The method of claim 8, wherein, when R4, R5 and R6 are part of a ring structure, the ring structure is a C_{1-22} alkyl ring or a C_{1-22} aromatic ring.

13. The method of claim 8, wherein said compound is selected from the group consisting of a monohydroxybenzoate, a dihydroxybenzoate and a trihydroxybenzoate.

14. The method of claim 8, wherein said compound is selected from the group consisting of 2,3-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid.

15. A method of prophylactically or therapeutically treating a cell, a tissue, an organ or an organism for cardiotoxicity, which method comprises administering to said cell, tissue, organ or organism a composition comprising an anti-cardiotoxicity effective amount of a compound of formula:



wherein R_1 = COO- or a precursor to COO-,

R_2 and R_3 = H, OH, or a precursor to OH, and

R_4, R_5 and R_6 = H, OH, a precursor to OH or a macromolecule or, together, R_4, R_5 and R_6 are part of a ring structure, with the proviso that, when R_1 = COO- and R_2 = OH or a precursor to OH, wherein said precursor to OH is $CH_3C(O)O$-, at least one of R_3-R_6 is other than H.

16. The method of claim 15, wherein said precursor to COO- is selected from the group consisting of a (i) cyano group, (ii) CH_2OH, (iii) CHO, (iv) COOR, wherein R = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via a threonine or a serine), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative, and (v) an amide, wherein said amide is

selected from the group consisting of CONR1 or CONR1R2, wherein R1 and R2 are selected from the group consisting of a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via an ε-amino group of lysine or a direct amide cap), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative.

17. The method of claim 15, wherein said precursor to OH is selected from the group consisting of an ester and OR, wherein R is a C_{1-22} alkyl or a C_{1-22} aryl.

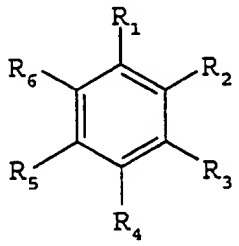
18. The method of claim 15, wherein, when R4, R5 or R6 is a macromolecule, the macromolecule is selected from the group consisting of a protein, a polysaccharide, a lipid, a lipopolysaccharide, and a nucleic acid derivative.

19. The method of claim 15, wherein, when R4, R5 and R6 are part of a ring structure, the ring structure is a C_{1-22} alkyl ring or a C_{1-22} aromatic ring.

20. The method of claim 15, wherein said compound is selected from the group consisting of a monohydroxybenzoate, a dihydroxybenzoate and a trihydroxybenzoate.

21. The method of claim 15, wherein said compound is selected from the group consisting of 2,3-dihydroxybenzoic acid , 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid.

22. A method of therapeutically treating a mammal for chemotherapeutic drug extravasation injury, which method comprises administering to said mammal a composition comprising an anti-chemotherapeutic drug extravasation injury effective amount of a compound of formula:



wherein R_1 = COO- or a precursor to COO-,

R_2 and R_3 = H, OH, or a precursor to OH, and

R_4, R_5 and R_6 = H, OH, a precursor to OH or a macromolecule or, together, R_4, R_5 and R_6 are part of a ring structure, with the proviso that, when R_1 = COO- and R_2 = OH or a precursor to OH, wherein said precursor to OH is $CH_3C(O)O$-, at least one of R_3-R_6 is other than H.

23. The method of claim 22, wherein said precursor to COO- is selected from the group consisting of (i) a cyano group, (ii) CH_2OH, (iii) CHO, (iv) COOR, wherein R = a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via a threonine or a serine), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative, and (v) an amide, wherein said amide is selected from the group consisting of CONR1 or CONR1R2, wherein R1 and R2 are selected from the group consisting of a C_{1-22} alkyl, a C_{1-22} aryl or a macromolecule selected from the group consisting of a protein (in which case binding to the protein is via an ε-amino group of lysine or a direct amide cap), a lipid, a polysaccharide, a lipopolysaccharide, and a nucleic acid derivative.

24. The method of claim 22, wherein said precursor to OH is selected from the group consisting of an ester and OR, wherein R is a C_{1-22} alkyl or a C_{1-22} aryl.

25. The method of claim 22, wherein, when R4, R5 or R6 is a macromolecule, the macromolecule is selected from the group consisting of a protein, a polysaccharide, a lipid, a lipopolysaccharide, and a nucleic acid derivative.

26. The method of claim 22, wherein, when R4, R5 and R6 are part of a ring structure, the ring structure is a C_{1-22} alkyl ring or a C_{1-22} aromatic ring.

27. The method of claim 22, wherein said compound is selected from the group consisting of a monohydroxybenzoate, a dihydroxybenzoate and a trihydroxybenzoate.

28. The method of claim 22, wherein said compound is selected from the group consisting of 2,3-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid.

FIG. 1

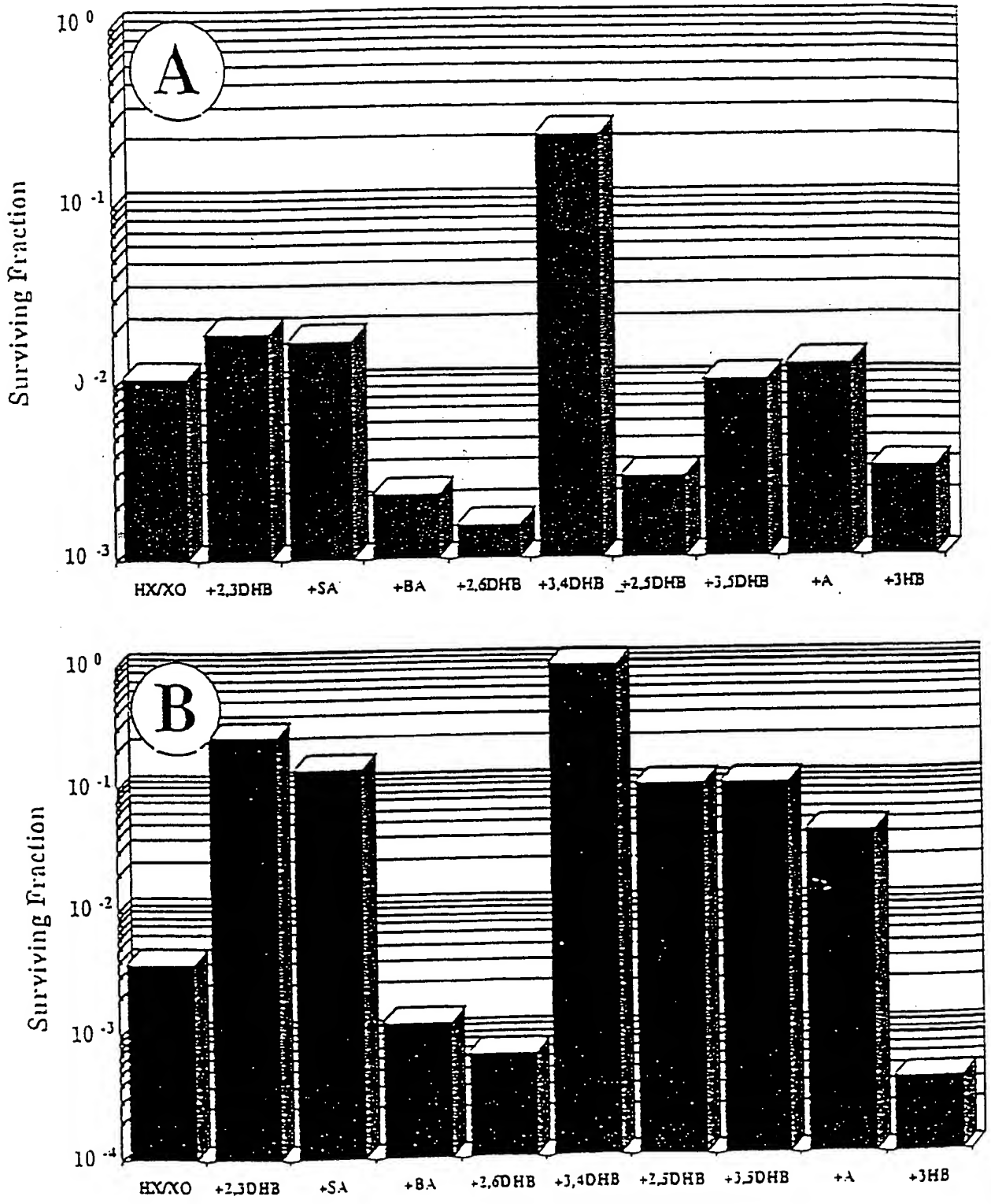

A
Surviving Fraction
10 0
10 -1
10 -2
10 -3
HX/XO
+2,3DHB
+SA
+BA
+2,6DHB
+3,4DHB
+2,5DHB
+3,5DHB
+A
+3HB
B
Surviving Fraction
10 0
10 -1
10 -2
10 -3
10 -4
HX/XO
+2,3DHB
+SA
+BA
+2,6DHB
+3,4DHB
+2,5DHB
+3,5DHB
+A
+3HB

FIG. 2

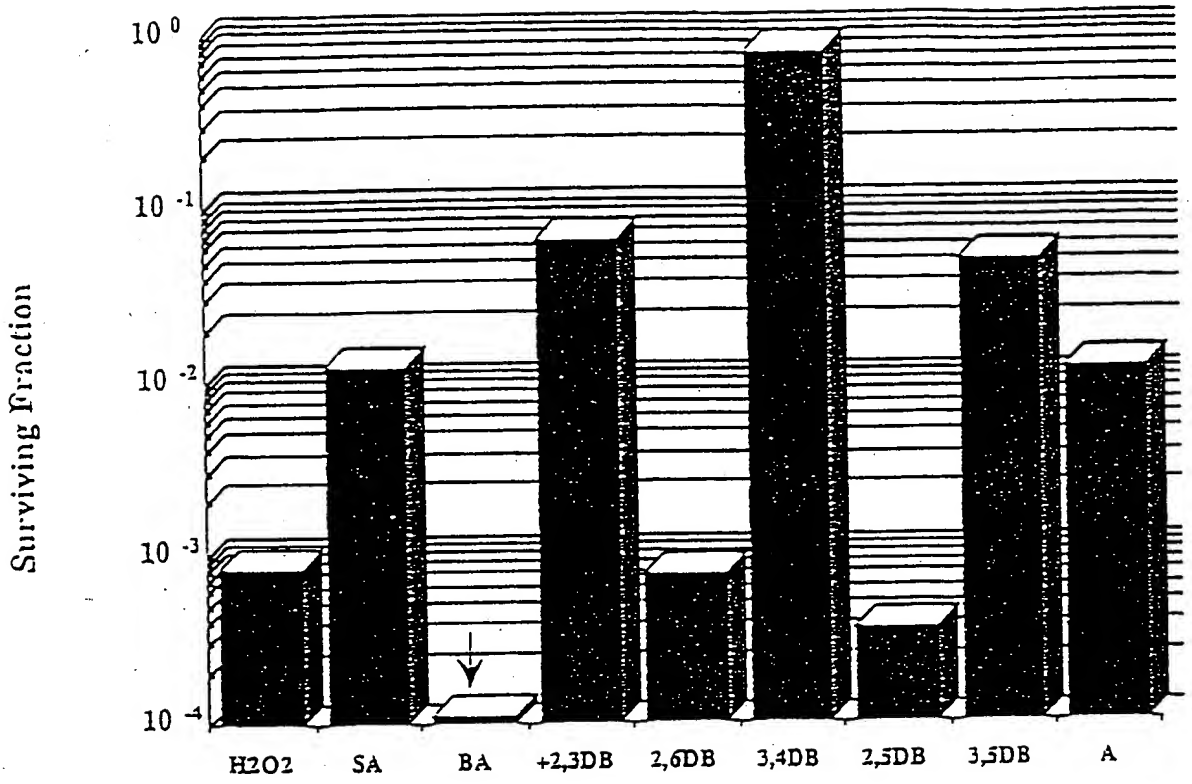

Surviving Fraction
10 0
10 -1
10 -2
10 -3
10 -4
H2O2
SA
BA
+2,3DB
2,6DB
3,4DB
2,5DB
3,5DB
A

FIG. 3

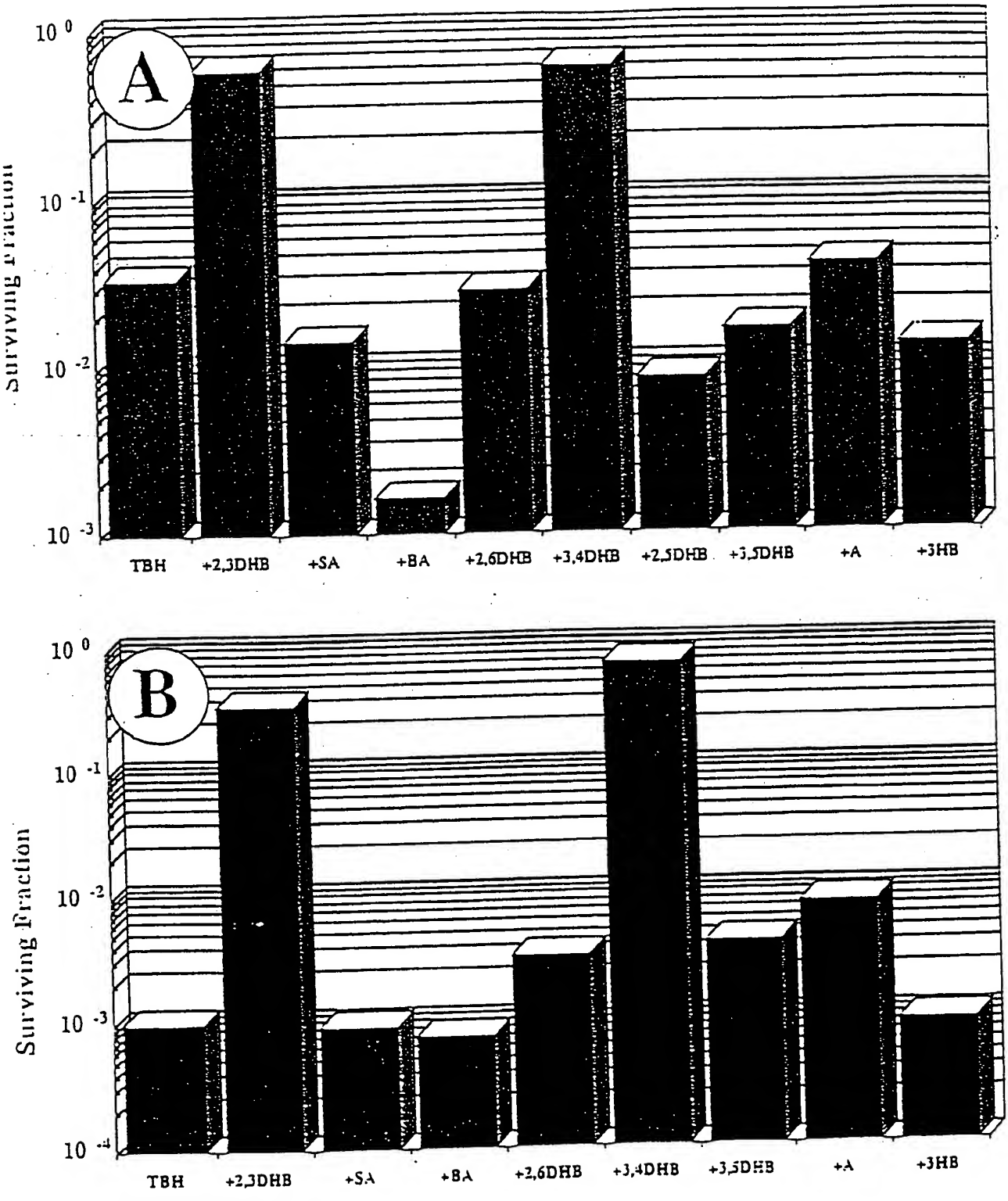

A
B
Surviving Fraction
10 0
10 -1
10 -2
10 -3
10 -4
TBH
+2,3DHB
+SA
+BA
+2,6DHB
+3,4DHB
+2,5DHB
+3,5DHB
+A
+3HB

FIG. 4

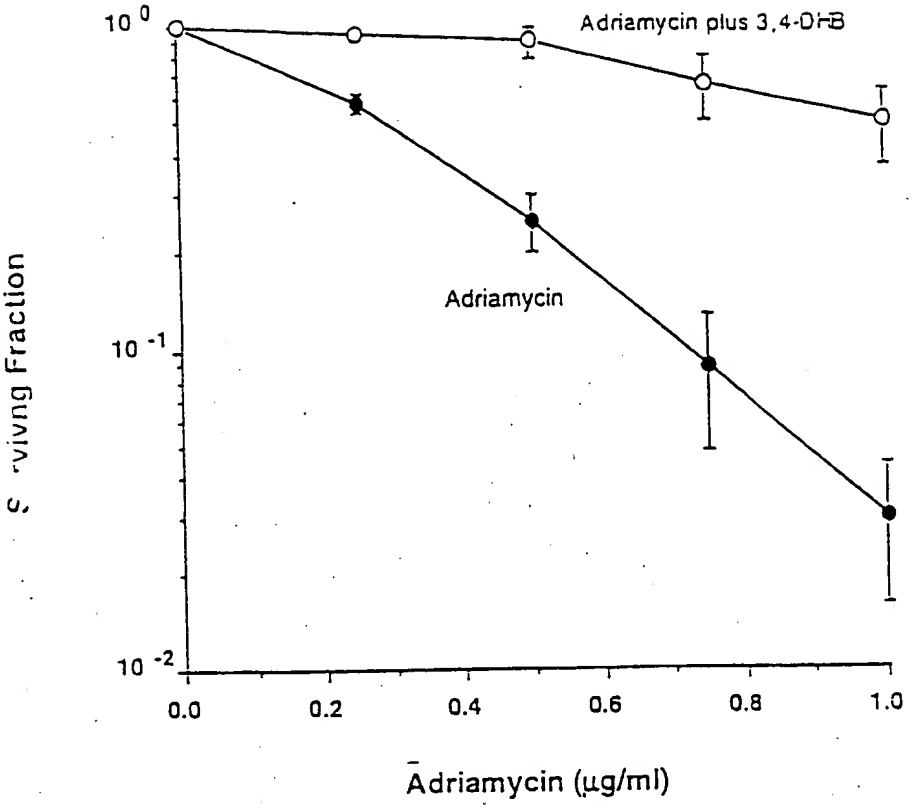


FIG. 4A

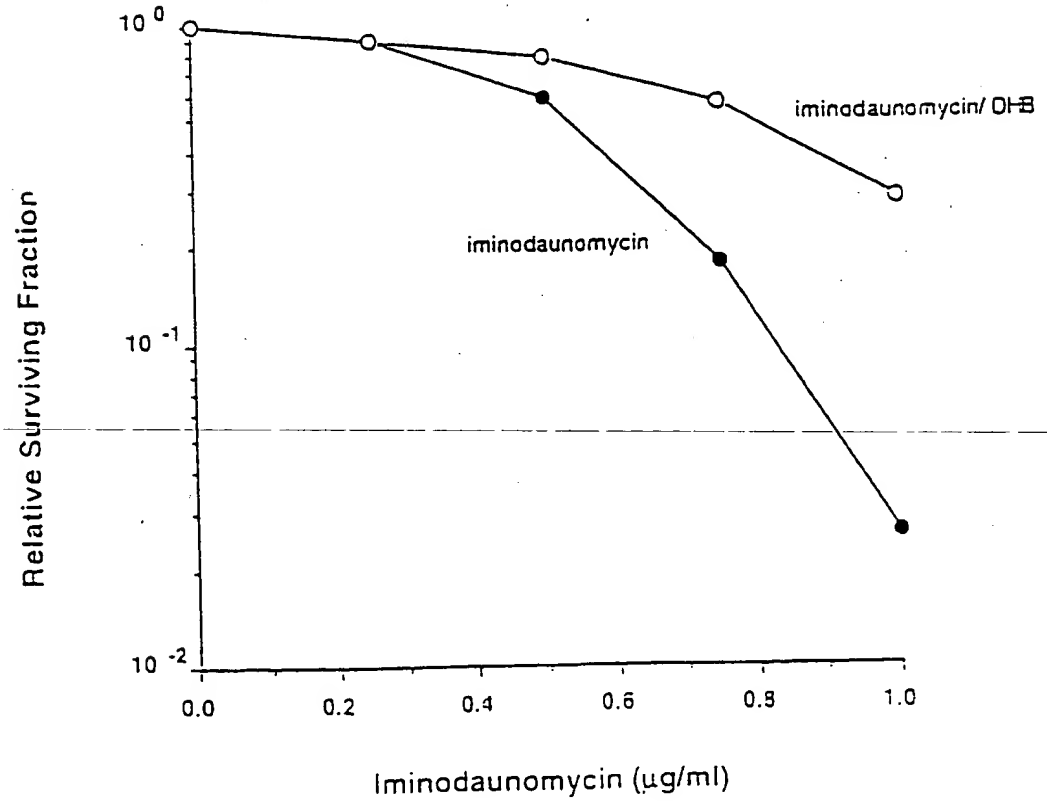


FIG.4B

FIG. 5

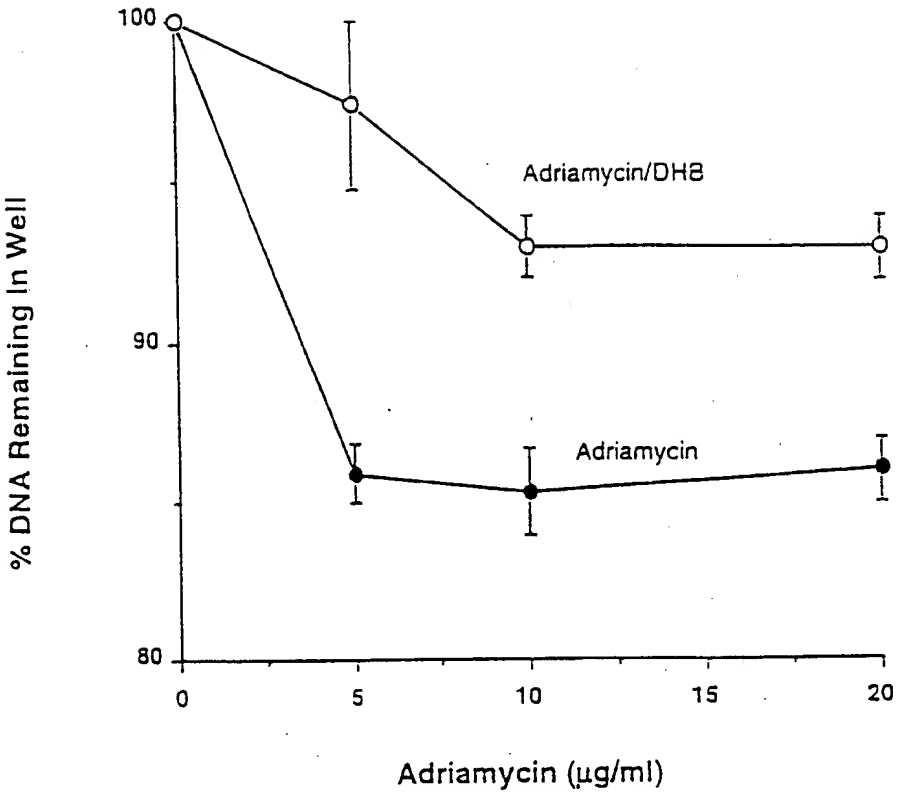

100
90
80
% DNA Remaining In Well
Adriamycin/DHB
Adriamycin
0
5
10
15
20
Adriamycin (µg/ml)

FIG. 6

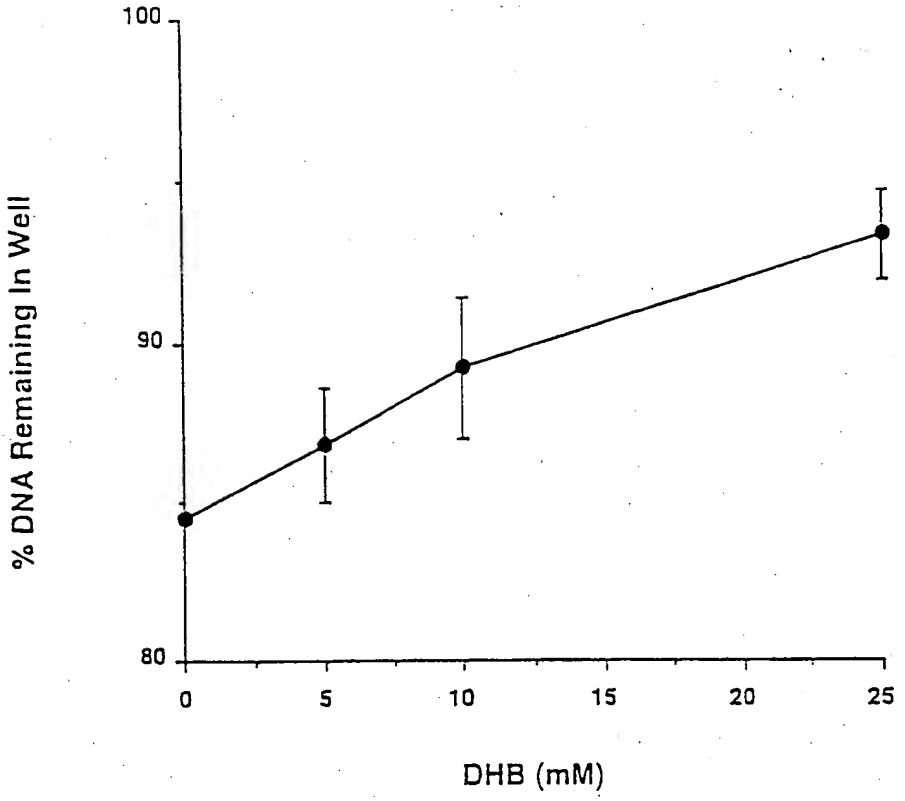
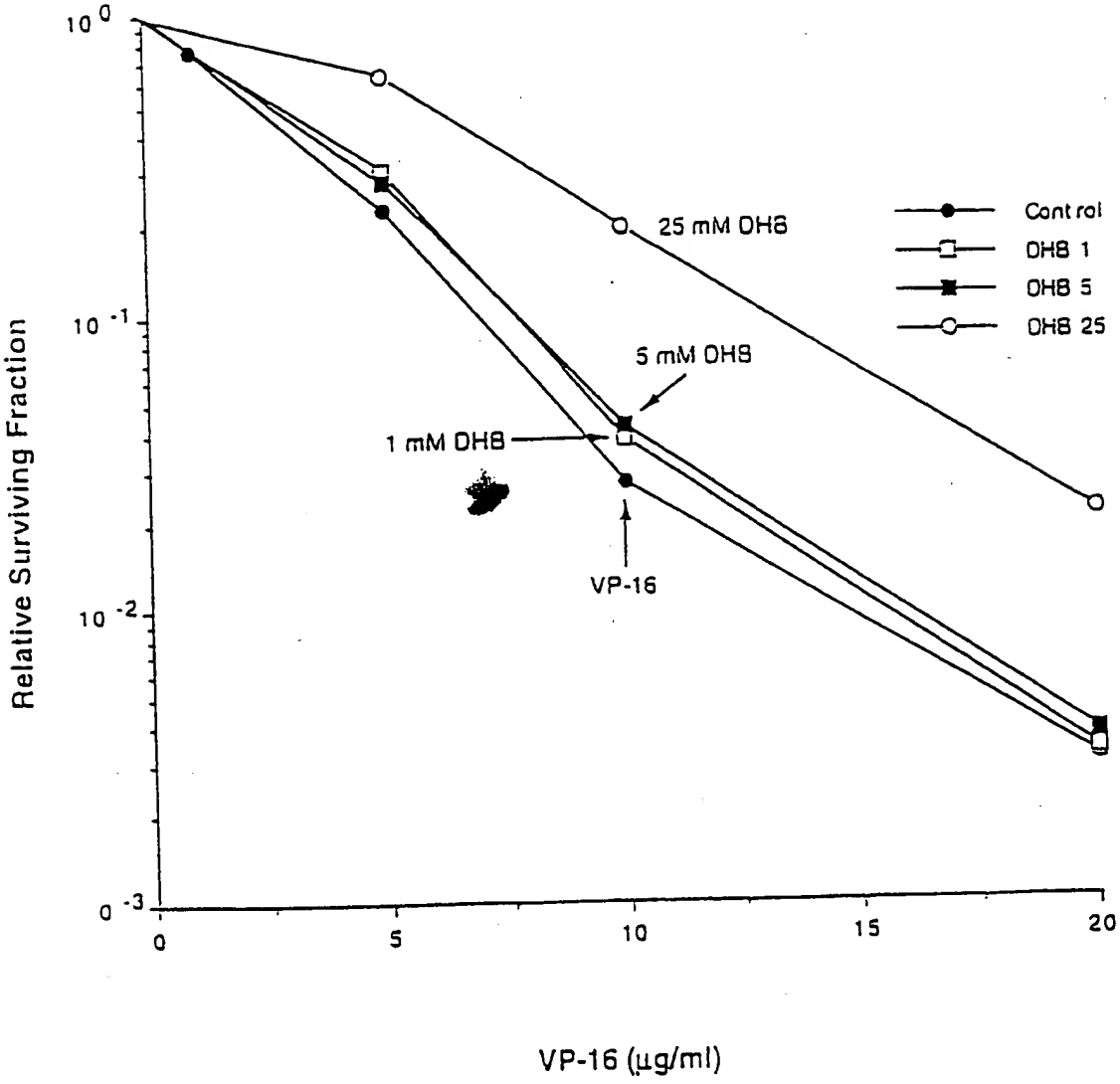


FIG. '7


Relative Surviving Fraction
10 0
10 -1
10 -2
0 -3
0
5
10
15
20
VP-16 (µg/ml)
25 mM OH8
5 mM OH8
1 mM OH8
VP-16
Control
OH8 1
OH8 5
OH8 25

INTERNATIONAL SEARCH REPORT

I. national Application No
PCT/US 98/05554

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/19 A61K31/605

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UEDA ET AL.: "A Comparison of Scavenging Abilities of Antioxidants against Hydroxyl Radicals" ARCH. BIOCHEM. BIOPHYS., vol. 333, no. 2, 1996, pages 377-384, XP002073681 see abstract; figures 1-4,6,9; table 1 ---	1-14
X	SAPPEY ET AL.: "NF-kappaB transcription Factor Activation by Hydrogen Peroxyde Can Be Decreased by 2,3-Dihydroxybenzoic Acid and Its Ethyl Ester Derivative" ARCH. BIOCHEM. BIOPHYS., vol. 321, no. 1, 1995, pages 263-270, XP002073682 see abstract see page 265, column 1, paragraph 5 - page 267, column 2, paragraph 1; figure 1 ---	1-14
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1998	17/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/05554

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEGRAFF ET AL.: "Protection against Adriamycin cytotoxicity and inhibition of DNA topoisomerase II activity by 3,4-dihydroxybenzoic acid" PROC. AM. ASSOC. CANCER RES., vol. 38, 1997, pages 22-23, XP002073683 see the whole document ---	1-14
X	HABTEMARIAM S.: "Modulation of Tumour Necrosis Factor-alpha-Induced Cytotoxicity by Polyphenols" PHYTOTHER. RES., vol. 11, no. 4, 1997, pages 277-280, XP002073684 see abstract see page 278, column 1, paragraph 1 - column 2, paragraph 1; figures 1-4 ---	1-14
X	SAGONE ET AL.: "OXIDATION OF SALICYLATES BY STIMULATED GRANULOCYTES: EVIDENCE THAT THESE DRUGS ACT AS FREE RADICAL SCAVENGERS IN BIOLOGICAL SYSTEMS" J. IMMUNOL., vol. 138, no. 7, 1987, pages 2177-2183, XP002073685 see abstract see page 2178, column 2, paragraph 7 - page 2181, column 1, paragraph 1; figures 1-3; tables 1-5 ---	1-7
X	WO 84 04676 A (RIET BARTHOLOMEUS VAN T;WAMPLER GALEN L; ELFORD HOWARD L) 6 December 1984 see table 7 ---	1,2
X	EP 0 310 126 A (HOFFMANN LA ROCHE) 5 April 1989 see page 21, line 27-40; claims 1-14; examples 1-164; tables 1-3 ---	1-6, 15-20, 22-27
X	D'AGNILLO ET AL.: "REDUCTION OF HYDROXYL RADICAL GENERATION IN A RAT HINDLIMB MODEL OF ISCHEMIA-REPERFUSION INJURY USING CROSSLINKED HEMOGLOBIN-SUPEROXIDE DISMUTASE-CATALASE" ART. CELLS, BLOOD SUS., AND IMMOB. BIOTECH., vol. 25, no. 1,2, 1997, pages 163-180, XP002073686 see abstract --- -/--	1-6,8-13

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/05554

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SATYAMOORTHY ET AL.: "POTENTIATION OF HYDROXYUREA CYTOTOXICITY IN HUMAN CHRONIC MYELOID LEUKEMIA CELLS BY IRON-CHELATING AGENT" LEUK. RES., vol. 10, no. 11, 1986, pages 1327-1330, XP002073687 see page 1327, column 1, line 1 - page 1328, column 1, paragraph 4; table 1 ---	1-14, 22-28
X	SHIH-CHEN ET AL.: "Mechanism of radiosensitizing effect of chloride ion on E. coli" INT. J. RADIAT. BIOL., vol. 49, no. 5, 1986, pages 799-808, XP002073688 see abstract; figures 4,5 ---	1-6,8-13
X	FLOYD ET AL.: "Hydroxyl Free Radical Mediated Formation of 8-Hydroxyguanine in Isolated DNA" ARCH. BIOCHEM. BIOPHYS., vol. 262, no. 1, 1988, pages 266-272, XP002073689 see abstract; figures 6,7 ---	1-6,8-13
X	SZEKERES L.: "Cardioprotection and anti-ischemic and cytoprotective drugs" BASIC RES. CARDIOL., vol. 82, no. Suppl. 2, 1987, pages 325-333, XP002073690 see abstract; tables 1,2,4 ---	1-21
X	CHANG ET AL.: "Inhibition of Platelet Activation and Endothelial Cell Injury by Polyphenolic Compounds Isolated from Lonicera japonica Thunb" PROSTAGLANDINS, LEUKOTRIENES AND ESSENTIAL FATTY ACIDS, vol. 45, no. 4, 1992, pages 307-312, XP002073691 see abstract see page 309, column 2, paragraph 3 - page 311, column 1, paragraph 2; figures 1-3; table 3 ---	1-14, 22-28
X	TSENG ET AL.: "Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes" CHEM. BIOL. INTERACT., vol. 101, no. 2, 1996, pages 137-148, XP002073692 see abstract; figures 1-6 --- -/--	1-14, 22-28

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/05554

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ULRICHOVA ET AL.: "CYTOPROTECTIVE EFFECTS OF PHENOLICS FROM COLCHICUM ON RAT HEPATOCYTES" FITOTHERAPIA, vol. 66, no. 5, 1995, pages 399-402, XP002073693 see page 401, paragraph 4 - page 402, paragraph 3; table 1	1-14

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 98/05554

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

In view of the large number of compounds, which are defined by the general definition in the indipendent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application. (See Guidelines, Chapter III, paragraph 2.3).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/05554

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8404676 A	06-12-1984	US 4623659 A	18-11-1986
		AU 589111 B	05-10-1989
		AU 3013084 A	18-12-1984
		CA 1339221 A	05-08-1997
		DK 13785 A	11-01-1985
		EP 0144396 A	19-06-1985
		FI 843681 A	24-11-1984
		JP 5078299 A	30-03-1993
		JP 5001780 B	11-01-1993
		JP 60501409 T	29-08-1985
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		US 4942253 A	17-07-1990
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		AU 2332488 A	06-04-1989
		CA 1328279 A	05-04-1994
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		PT 88634 A,B	01-10-1988
		US 5025036 A	18-06-1991
		US 5191108 A	02-03-1993